State Key Laboratory of Applied Organic Chemistry[1], Lanzhou University, Lanzhou; Key Laboratory for Natural Medicine of Gansu Province[2], Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, P.R. China

Sesquiterpenes with various carbon skeletons from *Ligularia virgaurea* spp. *oligocephala*

Quan-Xiang Wu[1], Qing-Yi Wei[1] and Yan-Ping Shi[1,2]

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Prof. Yan-Ping Shi, PhD. Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, People's Republic of China
shiyp@lzu.edu.cn and shiyp@lzb.ac.cn

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Two new 8,9-seco-cacalol-type sesquiterpene lactones, ligulolide C (**1** and **2**) and six known sesquiterpenes, adenostylide (**3** and **4**), cacalol (**5**), 1-hydroxy-2-(3′-pentenyl)-3,7-dimethylbenzofuran (**6**), 1β, 6α-dihydroxy-4(14)-eudesmene (**7**) and (+)-oplopanone (**8**), have been isolated from an extract of the whole plant of *Ligularia virgaurea* spp. *oligocephala*. The structures of **1** and **2** were confirmed by spectroscopic methods including 1D and 2D NMR spectra. A discussion of biogenesis of **1** and **2** was described. Cytotoxicity against selected cancer cells human promyelocytic leukemia (HL-60), human ovarian (HO-8910) and human lung epithehial (A-549) of compounds **1**–**8** were measured *in vitro*.

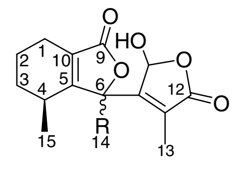
1. Introduction

As part of a research program on the bioactive constitutents of *Ligularia* species, we have investigated the whole plant of *L. virgaurea* spp. *oligocephala*, which has long been used as a traditional folk medicine for the treatment of stomachache and nausea (Wu et al. 1985). Except the eremophiloids from this plant as reported in the literature (Wu et al. 2004), two new 8,9-seco-cacalol-type sesquiterpene lactones, ligulolide C (**1** and **2**) and six known sesquiterpenes, adenostylide (**3** and **4**) (Kuroyanagi et al. 1985), cacalol (**5**) (Kuroyanagi et al. 1985), 1-hydroxy-2-(3′-pentenyl)-3,7-dimethylbenzofuran (**6**) (Jia et al. 1991), 1β,6α-dihydroxy-4(14)-eudesmene (**7**) (Hu et al. 1996) and (+)-oplopanone (**8**) (Li et al. 1990), were isolated from the extract of this species. In this study we report the isolation and structural elucidation of the new compounds, together with results about bioactivity testing toward the HL-60, HO-8910 and A-549 tumor cell lines. From the similarity in the structures of ligulolide C and adenostylide, a brief discussion of their biogenesis was described.

2. Investigations, results and discussion

From an extract of the whole plant of *L. virgaurea* spp. *oligocephala* two new 8,9-seco-cacalol-type sesquiterpene lactones (**1** and **2**) and six known sesquiterpenes **3**–**8** with the different carbon backgrounds were isolated. The known compounds **3**–**8** were identified by direct comparison of their spectral data (^{1}H NMR and ^{13}C NMR and DEPT 135) with literature values (Kuroyanagi et al. 1985; Jia et al. 1991; Hu et al. 1996; Li et al. 1990). Compounds **1** and **2** are sesquiterpene lactones, to the best of our knowledge, previously unreported.

Compounds **1** and **2** were obtained as a colorless amorphous powder with a molecular formula $C_{15}H_{18}O_5$ deter-



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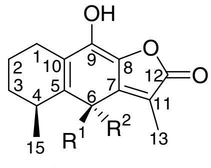
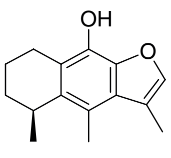
2 R=α-Me



3 R^1=Me, R^2=OH
4 R^1=OH, R^2=Me



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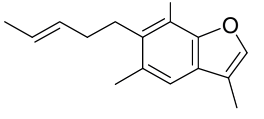

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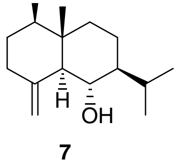

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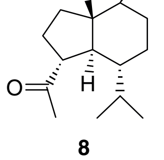

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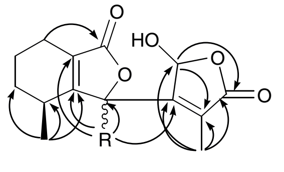
Table 1: NMR Spectral data of compounds 1 and 2[a]

No.	**1**		**2**	
	δ_H (J)	δ_C (DEPT)	δ_H (J)	δ_C (DEPT)
1a	2.23 m	20.41 t	2.28 m	20.41 t
1b	2.16 m		2.14 m	
2a	1.70 m	17.74 t	1.69 m	17.66 t
2b	1.64 m		1.63 m	
3a	1.68 m	30.95 t	1.67 m	30.95 t
3b	1.56 m		1.54 m	
4	2.56 m	28.54 d	2.54 m	28.58 d
5		169.56 s		169.46 s
6		86.71 s		86.97 s
7		160.43 s		160.28 s
8	5.85 s	97.81 d	5.85 s	97.99 d
9		172.87 s		172.81 s
10		126.36 s		126.17 s
11		126.05 s		125.93 s
12		169.82 s		169.66 s
13	2.09 s	13.03 q	2.08 s	12.97 q
14	1.87 s	24.49 q	1.86 s	24.06 q
15	1.27 d (7.2)	19.10 q	1.26 d (7.2)	19.15 q

[a] H NMR (400.16 MHz, δ values, TMS) coupling constants (Hz) are in parentheses and ^{13}C NMR (100.32 MHz, δ values, TMS) multiplication determined by DEPT, measured in $CDCl_3$

mined by the HR-ESI-MS ($[M + NH_4]^+$ $m/z = 296.1490$). Its IR spectrum showed the absorption bands for hydroxyl (3345 cm^{-1}), α,β-unsaturated-γ-lactone (1760, 1733 cm^{-1}) and double bond (1664 cm^{-1}) functions. The ^{13}C NMR spectrum (Table 1) of **1** and **2** showed 28 carbon resonance signals, two of which might overlap, so 30 carbon atoms might be expected. In the ^{13}C NMR and DEPT spectra (Table 1) of **1** and **2**, there were six methyl, six methylene, four methines and 14 quaternary carbons, including four α,β-unsaturated-γ-lactones ($\delta_C = 172.87$ (C-9), 126.36 (C-10), 169.56 (C-5); 172.81(C-9′), 126.17 (C-10′), 169.46 (C-5′); 169.82 (C-12), 126.05 (C-11), 160.43 (C-7); 169.66 (C-12′), 125.93 (C-11′), 160.28 (C-7′)) and four oxygenated carbons ($\delta_C = 86.71$ (C-6), 86.97 (C-6′), 97.81 (C-8) and 97.99 (C-8′)). From above properties of carbon pattern, the 30 carbons were considered to be 15 twins, which suggested compounds **1** and **2** were as epimeric sesquiterpenes each other. In the ^1H NMR spectrum (Table 1) of **1** and **2,** there are four singlet methyls ($\delta_H = 2.09$ (s, H-13), 2.08 (s, H-13′), 1.87 (s, H-14) and 1.86 (s, H-14′)), two doublet methyls ($\delta_H = 1.27$ (d, J = 7.2 Hz, H-15) and 1.26 (d, J = 7.2 Hz, H-15′)) like that of adenostylide, and two oxygenated methines ($\delta_H =$ 5.85 (2 H, s, H-8 and H-8′)), The ^1H NMR spectrum characteristic also supported the above reduction of epimeric sesquiterpenes. Extensive analyses of gCOSY and gHMBC (Fig.) data in $CDCl_3$ showed three rings. Ring A (C1−C5 and C15), very similar to that of adenostylide, was assembled on the basis of gCOSY correlations (H1, 1′-H2, 2′-H3, 3′-H4, 4′-H15, 15′ spin system) and gHMBC correlations (H15, 15′/C3, 3′, C4, 4′, C5, 5′). Ring B (C9−C10−C5−C6−O), analogical to ligulolide A except for the ester carbonyl group (Wu et al. 2004), was deduced on the basis of gHMBC correlations (H14,14′/C5, 5′, C6, 6′, C7, 7′, C10, 10′). Ring C (C7−C8−O−C12−C11 and C13), very similar to that of 6β-methacroyloxy-secomacrotolide acetate (Trendafilova et al. 2002), was established by gHMBC correlations (H13, 13′/ C6, 6′, C7, 7′, C8, 8′, C11, 11′, C12, 12′ and H8, 8′/C11, 11′, C12, 12′). At this point, the three partial rings (**A**−**C**) were assembled into a structure with the long-rang heteronuclear of correlations: H1, 1′/C9, 9′, H14, 14′/C7, 7′ and H13, 13′/C6, 6′. From these results, the structures of sesquiterpene **1** and its epimeric **2** could be elucidated as 8,9-seco-cacalol-type sesquiterpenes (Kuroyanagi et al. 1985), and named ligulolide C after the genus *Ligularia*. Like adenostylide, ligulolide C exists as a 1 : 0.9 mixture of epimers at C-6, i.e. (4R*, 6R*) and (4R*, 6S*). This mixture could not be separated by TLC (silica gel or R-18 silica gel) or HPLC, but gave a single spot or peak. The known naturally occurring adenostylide (**3** and **4**) may be the parent compound for the novel sesquiterpenes (**1** and **2**). Firstly adenostylide was oxygenated, and then the olefinic bond $\Delta^{8,9}$ was cleaved to yield an intermediate molecule (KIM), which changed into ligulolide C at the end by a couple of steps of changes including important lose a molecular H_2O reaction in internal molecule (Scheme).

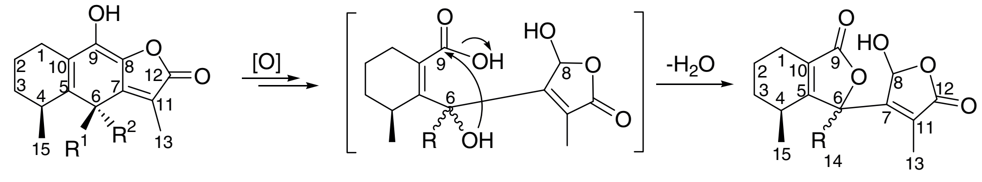


1 R=β-Me
2 R=α-Me

Fig: HMBC correlations of **1** and **2** (from H to C)

Sesquiterpenes (**1**–**8**) with various carbon skeletons were tested for their ability to inhibit human tumor HL-60, HO-8910 and A-549 cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Niu et al. 2002; Toume et al. 2004). As shown in Table 2, compared to etoposide (VP-16), compounds **1**–**5**, **7** exhibited no significant inhibitory effects with IC_{50} values over 100 μM and compounds **6** and **8** have low inhibitory activities against HL-60 cells. The IC_{50} value, 83.37 ± 2.77 μM demonstrated that compound **6** has low cytotoxicity against HO-8910 cells. Compounds **1**–**5**, **7** and **8** were inactive against HO-8910 cells and compounds **1**–**8** were also inactive against A-549 cells. However, the activities of compounds **1**–**8** were stronger against HL-60 than HO-8910 and A-549 cells.

Scheme



3 R¹=Me, R²=OH
4 R¹=OH, R²=Me

KIM

1 R=β-Me
2 R=α-Me

Table 2: Cytotoxic activities of compounds 1–8

Test substances	IC$_{50}$ (μM)		
	HL-60	HO-8910	A-549
1 and **2**	241.83 ± 4.58	>400	>400
3 and **4**	153.76 ± 29.98	>400	>400
5	117.50 ± 12.57	362.72 ± 5.55	319.93 ± 16.75
6	65.17 ± 6.57	83.37 ± 2.77	126.33 ± 6.50
7	253.16 ± 38.67	>400	>400
8	97.00 ± 0	>400	>400
Etoposide (VP-16)	2.82 ± 0.14	146.71 ± 35.03	>400

3. Experimental

3.1. Equipment

Melting points were determined with an X-4 Digital Display Micro-Melting point apparatus, and are uncorrected. Optical rotations recorded in $CHCl_3$ using a Perkin Elmer 241 polarimeter. UV spectra were measured on a Spect 50-UV/Vis instrument (Analytic Jena AG). IR spectra were measured on an FTS165-IR instrument (Bio-Rad, USA). ^{1}H NMR (400.13 MHz) and ^{13}C NMR (100.62 MHz) were recorded on a Varian INOVA-400 FT-NMR spectrometer (USA) in $CDCl_3$ with TMS as internal standard. HRMS was recorded on a Bruker APEX II. Silica gel (200–300 mesh) used for column chromatography and silica gel (GF$_{254}$) for TLC are supplied by the Qingdao Marine Chemical Factory in China. Spots were detected on TLC by visualization under UV light, or by spraying with 98% H_2SO_4-EtOH (V : V = 5 : 95) followed by heating at 110 °C.

3.2. Plant material

The plant material, *Ligularia virgaurea* spp. *oligocephala* Good (Compositae) was collected in Huzhu County, Qinghai province, P.R. China in August 2002 and was identified by adjunct Prof. Ji MA, Faculty of Pharmacy, First Military Medical University of PLA, Guangzhou, P.R. of China. A voucher specimen (NO. 2002001) has been deposited at Key Laboratory of Natural Medicine for Gansu province.

3.3. Extraction and isolation

The extraction procedure was as reported elsewhere (Wu et al. 2004). The EtOAc extract (75 g) was subjected to column chromatography on silica gel (1000 g) using petroleum ether (60–90°C) with increasing volumes of acetone (V : V = 50 : 1, 30 : 1, 15 : 1, 10 : 1, 7 : 1, 5 : 1, 3 : 1, 1 : 1, each about 3.0 L) as eluent. Fractions were examined by TLC and combined to afford 8 pooled fractions (1A–1H). Fraction 1B (0.9 g, 3 L) was further fractionated on a silica gel column (20 g) eluting with petroleum ether-acetone (30 : 1, 800 mL) to give two fractions (fr.1B1, 200 mL and fr.1B2, 200 mL). Fr.1B1 (0.35 g) was further subjected to column chromatography on silica gel (10 g) eluting with petroleum ether-ethyl ether (30 : 1, 460 mL) to obtain compound **5** (65 mg, 200 mL). Fr.1B2 (0.38 g) was further subjected to column chromatography on silica gel (12 g) eluting with petroleum ether-ethyl ether (30 : 1, 500 mL) to obtain compound **6** (69 mg, 220 ml). Fraction 1C (4.2 g) was further fractionated on a silica gel column (90 g) eluting with petroleum ether-EtOAc (8 : 1, 1100 mL) to give three fractions (fr.1C1, 500 mL, fr.1C2, 200 mL, fr.1C3, 120 mL, and fr. 1C4, 280 mL). Fr.1C1 (2.1 g) was further subjected to column chromatography on silica gel (75 g) eluting with petroleum ether-acetone (8 : 1, 900 mL) to obtain a mixture of **3**, and **4** (90 mg, 430 mL). Fr.1C2 (0.8g) was further subjected to column chromatography on silica gel (30 g) eluting with $CHCl_3$-acetone (60 : 1, 740 mL) to obtain compound **7** (6.5 mg, 80 mL). Fr.1C3 (0.65 g) was further subjected to column chromatography on silica gel (27 g) eluting with $CHCl_3$-EtOAc (20 : 1, 320 mL) to obtain compound **8** (4.0 mg, 50 mL). Fr.1C4 (0.50 g) was further subjected to column chromatography on silica gel (24 g) eluting with $CHCl_3$-acetone (20 : 1, 200 mL) to obtain compound mixture **1**, **2** (20.6 mg, 50 mL).

3.3.1. Compounds 1 and 2

Colorless power, IR (Film): $\nu_{max} = 3345$, 2938, 1760, 1733, 1664, 1447, 1382, 1315, 1268, 1155, 1120, 1064, 1009, 970, 785, 741 cm^{-1}. UV (MeOH): λ (log ε) = 208.0 (5.65) nm. HR-ESI-MS: revealed *m/z* = 296.1490, requires m/z = 296.1492 for $C_{15}H_{22}O_5N$. ^{1}H and ^{13}C NMR: see Table 1.

3.3.2. Compounds 3–8

All as colorless needles. For melting points and ^{1}H NMR and ^{13}C NMR data see the literature (Kuroyanagi et al. 1985; Jia et al. 1991; Hu et al. 1996; Li et al. 1990). $[\alpha]_D^{18}$: +9° (c 0.31, $CHCl_3$) for mixture **3** and **4**, +8° (c 1.08, $CHCl_3$) for **5**, +2° (c 0.35, $CHCl_3$) for **6**, +42° (c 0.16, $CHCl_3$) for **7**, −8° (c 0.31, $CHCl_3$) for **8**. Additionally, copies of original spectra (^{1}H NMR, ^{13}C NMR and DEPT) are available from the author of correspondence.

3.3.3. Tests of cytotoxicity against tumor HL-60, HO-8910 and A-549 cells

A MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was performed in 96-well culture plates. The assay is based on reduction of MTT by the mitochondrial dehydrogenase of viable cells to yield a blue formazan product that can be measured spectrophotometrically. In the experiment, the negative controls were isochoric normal saline, 1% DMSO or 0.1% DMSO; positive control was VP-16 at concentrations of 2.5, 5, 10, and 20 μM. HL-60, HO-8910 and A-549 cells at a log phase of their growth cycle (1 × 10^5 cell/mL) were added to each well (90 μL/well), then treated in four replicates at various concentrations of the drugs with six vacant reference wells set in one plate (100 μL cultured media in each well) and incubated for 44 h at 37 °C in a humidified atmosphere of 5% CO_2. After 44 h, 10 μL of MTT solution (5 mg/mL) was added to each well, which was incubated for another 4 h, after which a solution of 10% sodium dodecyl sulfate (SDS) was added to each well (100 μL/well). Twelve hours later at room temperature, the optical density (O.D.) of each well was recorded on an ELISA reader (Bioteck EL-340) at one wavelength of 570 nm. The results were listed in the Table 2.

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